Nucleation and growth phases in the polymerization of coat and scaffolding subunits into icosahedral procapsid shells

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ABSTRACT The polymerization of protein subunits into precursor shells empty of DNA is a critical process in the assembly of double-stranded DNA viruses. For the well-characterized icosahedral procapsid of phage P22, coat and scaffolding protein subunits do not assemble separately but, upon mixing, copolymerize into double-shelled procapsids in vitro. The polymerization reaction displays the characteristics of a nucleation limited reaction: a paucity of intermediate assembly states, a critical concentration, and kinetics displaying a lag phase. Partially formed shell intermediates were directly visualized during the growth phase by electron microscopy of the reaction mixture. The morphology of these intermediates suggests that assembly is a highly directed process. The initial rate of this reaction depends on the fifth power of the coat subunit concentration and the second or third power of the scaffolding concentration, suggesting that pentamer of coat protein and dimers or trimers of scaffolding protein, respectively, participate in the rate-limiting step.

INTRODUCTION

A ubiquitous structural theme in the organization of spherical viruses is the formation of closed shells of protein subunits displaying icosahedral symmetry (Caspar and Klug, 1962; Rossmann, 1984). The three-dimensional structure of protein shells has been determined to atomic resolution for members of at least five families of viruses, including single-stranded RNA (reviewed by Rossmann and Johnson, 1989), single-stranded DNA (McKenna et al., 1991; Tsao et al., 1991), and double-stranded DNA (dsDNA) containing virions (Liddington et al., 1991). In these capsids, identical protein subunits form different types of intersubunit contacts in the final structure (Rayment et al., 1982; Rossmann, 1984; Silva and Rossmann, 1987). Determining the sequence of steps by which the capsid subunits interact during assembly is critical to ultimately understanding the controlled conformational switching required for high fidelity capsid assembly.

For organelles with helical symmetry, including microtubules, flagella, and tobacco mosaic virus, kinetic analyses have differentiated subunit polymerization into nucleation, propagation, and termination steps (Butler and Klug, 1971; Voter and Erickson, 1984; Namba and Stubbs, 1986; Raghavendra et al., 1986, 1988). Recognition of the nature of these steps led investigators to search for and identify specific effectors of initiation.

In the case of icosahedral structures, only limited descriptions of the polymerization pathways are available. For the best studied systems, the T = 3 RNA containing viruses, nucleation of assembly is likely to occur by the binding of coat protein dimers to the RNA, with growth proceeding by the addition of dimers to this nucleation complex. The number of coat protein dimers comprising the initiation complex remains in dispute (Sorger et al., 1986; Silva and Rossmann, 1987). For the T = 7 polyoma virus, the capsid appears to be assembled by the direct association of 72 stable pentamers of coat protein with the dsDNA (Salunke et al., 1986; Liddington et al., 1991). The picornaviruses are also believed to be assembled by the association of pentameric substructures (Rueckert et al., 1969; Rueckert, 1991).

For many dsDNA viruses, including adenoviruses (Morin and Boulanger, 1984), herpesviruses (O'Callaghan et al., 1977; Ladin et al., 1982; Preston et al., 1983; Baker et al., 1990; Newcomb and Brown, 1991), and bacteriophages (Showe and Black, 1973; King and Casjens, 1974; Casjens and King, 1975; Black and Showe, 1983), the coat protein subunits do not polymerize directly with the DNA to form the viral capsid. Instead, a precursor shell of coat protein empty of DNA but containing scaffolding proteins is constructed (Casjens and King, 1974; Black and Showe, 1983). The viral DNA is subsequently packaged into the procapsid. The scaffolding proteins are removed before DNA packaging (Casjens and King, 1974; King and Casjens, 1974; Murialdo and Becker, 1978; Black and Showe, 1983), and the capsid lattice expands via changes in the coat subunit conformations and interactions (Earnshaw et al., 1976; Casjens, 1979; Steven et al., 1990, 1991). Thus, it is the procapsid, rather than the mature capsid, that is the direct product of the subunit polymerization process. The mechanisms by which the assembly process is initiated and subsequent growth occurs are unclear.

The procapsid of *Salmonella typhimurium* phage P22 is composed of ~420 copies of the gene 5 encoded coat protein of 47,600 D with an inner core composed of

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0006-3495/93/03/824/12 $2.00

Biophys. J. © Biophysical Society
Volume 64 March 1993 824-835

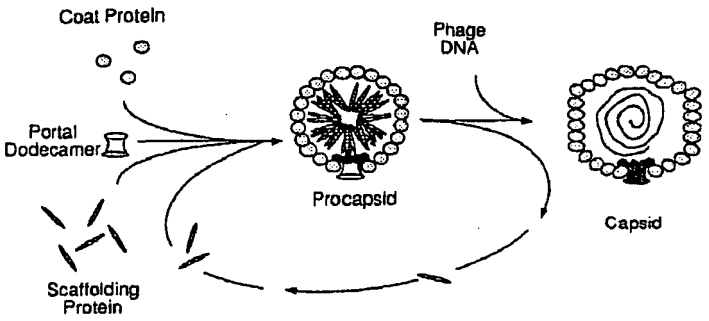

FIGURE 1 The morphogenesis of bacteriophage P22. Approximately 420 molecules of coat protein, ~300 molecules of scaffolding protein, and 12 molecules of the portal protein polymerize into a double-shelled procapsid in which the scaffolding protein forms an internal core. The replicated DNA is packaged by a headful mechanism into the procapsid, resulting in the release of scaffolding protein and expansion of the coat protein lattice yielding the capsid. The freed scaffolding protein can recycle and participate in further rounds of assembly.

~300 copies of the 33,000 D gene 8 encoded scaffolding protein (Botstein et al., 1973; King and Casjens, 1974; Eppler et al., 1991). A dodecameric ring of the portal protein is located at one vertex and forms a channel through which the DNA is packaged (Bazinet et al., 1988). Before DNA packaging, the scaffolding subunits exit from the procapsid without proteolytic cleavage and participate in further rounds of procapsid assembly (Fig. 1). Although for the prolate phage the portal protein plays an important role in form determination (Black and Showe, 1983; Kellenberger, 1990; Guo et al., 1991), in P22 the presence of the portal protein is not essential for form determination nor does its absence affect the rate of assembly (Bazinet and King, 1988). As a result, closed icosahedral procapsid-like shells can be assembled from scaffolding and coat species alone (Fuller and King, 1982; Prevelige et al., 1988). Since neither scaffolding nor coat protein is covalently altered during assembly, it has been possible to isolate these subunits for use as precursors for procapsid assembly in vitro (Fuller and King, 1982; Prevelige et al., 1988).

The polymerization of coat and scaffolding subunits into procapsids in vitro displays similar regulation as assembly observed in vivo: neither subunit polymerizes by itself. The purified proteins exist in solution predominantly as 3.2 S coat monomers and 2.8 S scaffolding monomers as determined by gel chromatography and sucrose gradient centrifugation (Prevelige et al., 1988). Simple mixing of the solutions of the purified subunits without change in solvent conditions resulted in the formation of closed double-shelled procapsids sedimenting at ~240 S (Prevelige et al., 1988; Thomas and Prevelige, 1991).

The ability to initiate the assembly reaction by direct mixing of the component species opens the door for the application of kinetic techniques to study the assembly reaction. In this article we report evidence that the overall course of polymerization is well directed and follows a slow nucleation-fast growth model. The kinetics of the initial rate of reaction provides evidence regarding the size of the nucleation complex.

MATERIAL AND METHODS

Preparation of protein subunits

Subunits of coat and scaffolding protein were prepared by dissociation of procapsids as previously described (Prevelige et al., 1988; Thomas and Prevelige, 1991). The released scaffolding protein was purified by chromatography through carboxymethyl-Sepharose and diethylaminoethyl columns concentrated by precipitation with ammonium sulfate and exhaustively dialyzed (Thomas and Prevelige, 1991). Purified scaffolding protein was stored frozen as a stock solution at 5 mg/ml. The same preparation, though not the same aliquot of scaffolding protein, was used for all the kinetic experiments reported. The experiments involving glutaraldehyde cross-linking and electron microscopy were performed with a different preparation of scaffolding protein that was stored at 6.1 mg/ml.

Coat protein subunits were prepared by dissociation of shells and renaturation as previously described (Prevelige et al., 1988). The shells were dissociated by adding an equal volume of 6 M GuHCl, resulting in a final concentration of 3 M GuHCl, and applied to a BioGel A 0.5-m (BioRad Laboratories, Richmond, CA) column. Fractions from the included peak were pooled and the concentration adjusted such that the resultant concentration was ~1.2 mg/ml. Coat protein concentrations higher than this resulted in aggregation during refolding. The protein was refolded by dialysis overnight in Spectrapor 3 dialysis tubing against 2 × 1 l of buffer B (50 mM tris(hydroxymethyl)-aminomethane, 25 mM NaCl, 2 mM ethylenediaminetetraacetate, pH 7.6) at 4°C. Attempts to concentrate the refolded protein by a wide variety of techniques resulted in aggregation.

After dialysis, the protein subunits were removed from the dialysis tubing and maintained at 4°C. The scattering at 350 nm was typically <0.05 OD units. The stock solution of protein was maintained at 4°C and discarded at the end of the day. This preparative protocol resulted in coat protein subunits that were >90% biologically active and remained monomeric in solution (Prevelige et al., 1988). All experiments were performed in buffer B.

In vitro assembly reactions

For the kinetic experiments where the coat protein concentration was varied, the coat protein and buffer were removed from ice, mixed in an Eppendorf tube, and equilibrated at room temperature for 5 min. Scaffolding protein was added, and the contents of the tube were transferred to a thermostatted cuvette. The dead time between adding the scaffolding protein and the first data point was typically 30 s. Alternatively, coat protein was temperature equilibrated in the thermostatted cuvette holder, and scaffolding protein added with mixing was accomplished by inversion. The dead time for this protocol was typically 15 s.

The experiments in which the scaffolding protein concentration was varied were performed by removing coat protein from ice, adding the appropriate volume of buffer, and equilibrating the sample for 5 min in a thermostatted cuvette. The 5-mg/ml stock solution of scaffolding protein was diluted to 0.5 mg/ml with buffer. Temperature equilibrated scaffolding protein from the 0.5-mg/ml stock solution was added directly to the cuvette and the sample mixed by repeated inversion of the cuvette. The dead time for this experiment was typically 15 s.

Scattering was monitored in a spectrophotometer (Response; Gilford Instruments Laboratories, Inc., Oberlin, OH) equipped with a thermal cuvette holder and 1-cm cells at a wavelength of 250 nm. The sample volume was 300 μl. Data was acquired at 20-s intervals for the coat protein experiments and 3.75-s intervals for the scaffolding protein experiments.

Electron microscopy of assembly reactions

Carbon films were prepared by evaporation of carbon onto freshly cleaved mica. The films were floated off and picked up on copper grids. Negative staining was on the grid by the method of Huxley and Zubay (1960) using 2% (wt/vol) uranyl acetate. Samples were examined at 80 kV with an electron microscope (model 100B; JEOL U.S.A. Inc., Peabody, MA).

Critical concentration determination

Assembly reactions were carried out at coat protein concentrations of 0.2, 0.4, 0.6, 0.8, and 1.0 mg/ml, and a scaffolding protein concentration of 1 mg/ml at 20°C. The reactions were monitored for 5 h by turbidity at 350 nm in a thermostatted spectrophotometer. Parallel reactions that lacked scaffolding protein were maintained at room temperature. All reactions were then centrifuged in an ultracentrifuge using a rotor (models TL-100 and TL-100.3, respectively; Beckman Instruments, Fullerton, CA) and Eppindorf tubes at 40,000 rpm, 30 min, 20°C, and 200 μl of the 300 μl volume were carefully removed. Control experiments with purified procapsids demonstrated that procapsids are contained completely in the pellet using this protocol. The concentration of coat protein in the supernatant was determined by quantitative sodium dodecyl sulfate (SDS) gel electrophoresis and densitometery relative to the control that lacked scaffolding protein.

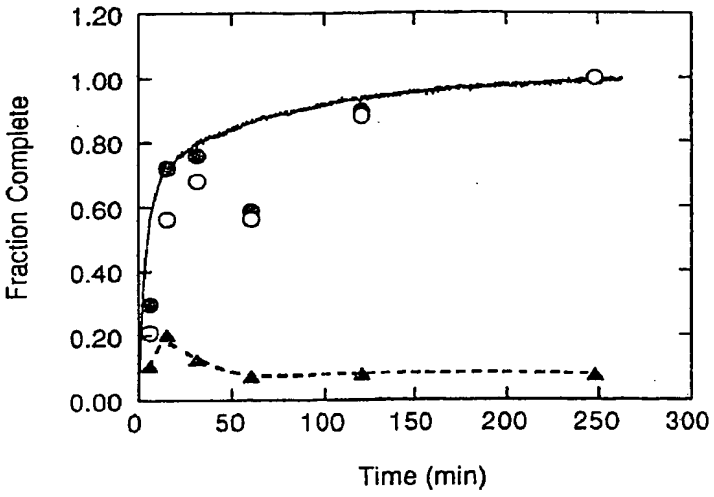

FIGURE 2 Fraction complete determined by electron microscopy and turbidity. The fractional increase in turbidity is represented by the solid line. The number of fully formed procapsids (○), partially formed procapsids (▲), and the sum of both forms (●) was determined relative to a phage counting standard at various time points during the reaction. The fraction completed was determined using the respective final value for the fully formed procapsids and the sum of both forms. The fraction partials is calculated relative to the sum of both forms. Procapsids were defined as particles presenting as a completely closed circle of density, displaying a diameter estimated visually to be that of a procapsid. Any particle that was not a completely closed circle of density was scored as a partial shell. Approximately 300 phage were counted per time point. Assembly reactions were initiated by adding coat protein equilibrated to 20°C to a thermostatted cuvette scaffolding protein. The final concentration of both proteins was 0.77 mg/ml. The turbidity was monitored at 350 nm. At the indicated times, the sample chamber was opened, an aliquot of the reaction mixture removed, mixed with phage, and applied to a carbon-coated grid.

RESULTS

Overall kinetics of procapsid assembly in vitro

Incubation of purified coat and scaffolding subunits together results in an increase in turbidity with time. Analysis of the products by electron microscopy and sucrose gradient centrifugation showed that at late times the increase in turbidity correlated with procapsid formation (Prevelige et al., 1988). To determine if the increase in turbidity corresponded to the gradual growth of procapsids in solution (in which large numbers of assembly intermediates would be present) or if the reaction displayed the nucleation-limited, fast-growth characteristics seen for helical polymerization (Oosawa and Kasai, 1962; Voter and Erickson, 1984; Wegner and Engel, 1975; Tobacman and Korn, 1983; Frieden and Goddette, 1983), we followed the assembly reaction simultaneously by both turbidity and electron microscopy (Fig. 2).

The turbidity profile displayed an initial fast rise followed by a prolonged gradual increase. At this protein concentration there was no apparent lag between the beginning of the reaction and the onset of the turbidity change. Under these conditions neither the coat or scaffolding protein individually displayed any increase in turbidity (data not shown).

At the indicated times the spectrophotometer was opened and the reaction sampled for electron microscopy. At all times the only large structures detected by electron microscopy were procapsids, a small number of particles that appeared to be partial shells, and the added marker phage. We would expect to be able to detect oligomers that were on the order of hexamers or larger. The appearance of predominantly completed shells and only a small number of intermediates throughout the reaction suggests that the reaction may be nucleation limited. The kinetics of formation of the procapsids plus partial shells followed the increase in turbidity, demonstrating that both procapsids and partial procapsids contributed to the turbidity approximately in proportion to their number and that all the species that contributed significantly to the turbidity were detected by electron microscopy. No evidence for the rapid formation of large polymers of subunits in morphological forms other than shell-like particles (i.e., sheets) was observed.

If rate-limiting nucleation is followed by relatively rapid growth, then there should be very few intermediates present during an assembly reaction. Although the electron microscopy results suggest that there are few intermediates present, it is possible that intermediates were dissociating during the preparation of the grids, and the number of partial shell intermediates observed was not representative of their concentration in solution.

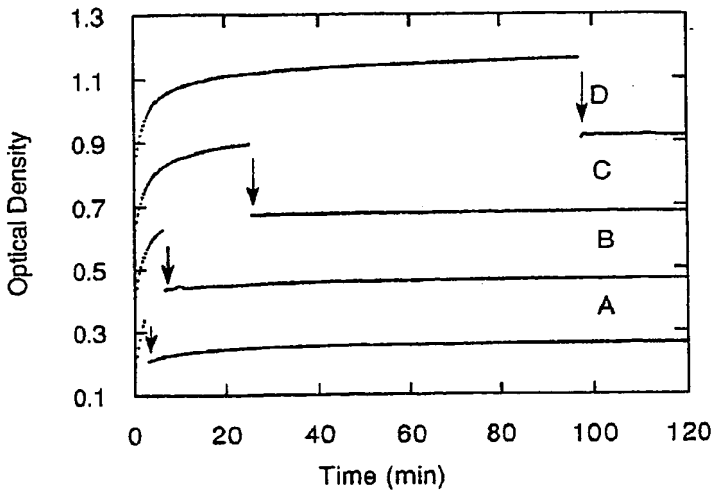

FIGURE 3 Effect of dilution on turbidity during assembly. Four assembly reactions were initiated by adding temperature equilibrated scaffolding protein from a 5-mg/ml solution to coat protein equilibrated at 20°C. The final concentration of both proteins was 1 mg/ml. The turbidity was monitored at 310 nm, whereas the assembly reactions were diluted two times by the introduction of an equivalent volume of temperature equilibrated buffer at 2 min (*A*), 7 min (*B*), 29 min (*C*), and 97 min (*D*).

Rapid dilution of the assembly reaction would dissociate unstable intermediates and procapsids, in which case the scattering should decrease in excess of the dilution effect. To directly test this hypotheses, we diluted assembly reactions with buffer at various points during assembly and monitored the scattering (Fig. 3).

Assembly reactions were performed with initial concentration of 1 mg/ml each of coat and scaffolding protein. The reactions were diluted by half with reaction buffer at 3, 7, 25, and 97 min. The largest deviation of the observed value after dilution from the calculated value occurred at 7 min and was 3%. This suggests that the number of intermediates seen by microscopy was representative of the number in solution.

Partial shell intermediates

The partial shells observed in the experiment of Fig. 2 were candidates for intermediates in assembly. However, at late times the low level of intermediates would be difficult to distinguish in a field of completed particles. Therefore, we attempted to observe reactions at early times where the background from finished particles would be minimal. To optimally visualize putative partial shell assembly intermediates, the protocol was altered. Previous results using sucrose gradient centrifugation (Prevelige et al., 1988) together with the dilution experiment described above suggested that the few assembly intermediates present may be unstable upon dilution. We therefore attempted to stabilize structural intermediates by glutaraldehyde cross-linking. To further quench nucleation proceeding within the sample and to reduce the amount of unassembled protein in the background, the assembly reaction was diluted 10-fold before application to the sample grids.

Fig. 4 shows typical fields at early times during the assembly reaction. Neither the coat or scaffolding protein control samples individually revealed any recognizable structures. The first grid of the reaction mixture was prepared after 10 s of reaction and no structures were observed (data not shown). Organized complexes could be seen at 30 s (*A*). The "structures" appear as small regions of high capsomere density. There was no easily discernable symmetry at this level of resolution.

After 1 min there were incompletely formed procapsid-like particles (*B*). These particles were from one half to three quarters formed and displayed appropriate curvature. At 2 min the species observed completely formed properly dimensioned procapsids. These data suggest that growth occurs on the surface of the shell with the protein subunits maintaining the correct curvature as they add. There was no significant population of "off pathway" particles, suggesting a highly directed assembly process.

The critical concentration

In survey experiments, we determined the range of coat protein concentrations over which assembly occurred. At coat protein concentrations below ~0.3 mg/ml, there was no increase in turbidity even after 3 h. This suggested that 0.3 mg/ml is the critical concentration required for assembly. The presence of a critical concentration indicates a requirement for a distinct nucleation step (Oosawa and Kasai, 1962). At total protein concentrations below the critical concentration, there should be no polymerization, whereas at all protein concentrations exceeding the critical concentration, there should be an equilibrium between monomers and polymer in which the concentration of the monomers in solution equals the critical concentration.

To further investigate this possibility, we performed assembly reactions at 20°C containing 0.2, 0.4, 0.6, 0.8, and 1.0 mg/ml of coat protein and 1.0 mg/ml scaffolding protein (Fig. 5 *A*). Parallel reactions lacking scaffolding protein were maintained at room temperature. At a concentration of 0.2 mg/ml there was no assembly, but at a concentration of 0.4 and above assembly was observed. After allowing 5 h for reaction, the samples were fractionated by centrifugation and the concentration of soluble (<150 S) coat protein determined by quantitative SDS gel electrophoresis relative to sample that lacked scaffolding protein (Fig. 5 *B*). At an initial coat protein concentration of 0.2 mg/ml in the presence of scaffolding protein, all the protein was found in the supernatant and no assembly was observed by turbidity. At higher concentrations where assembly was observed, the

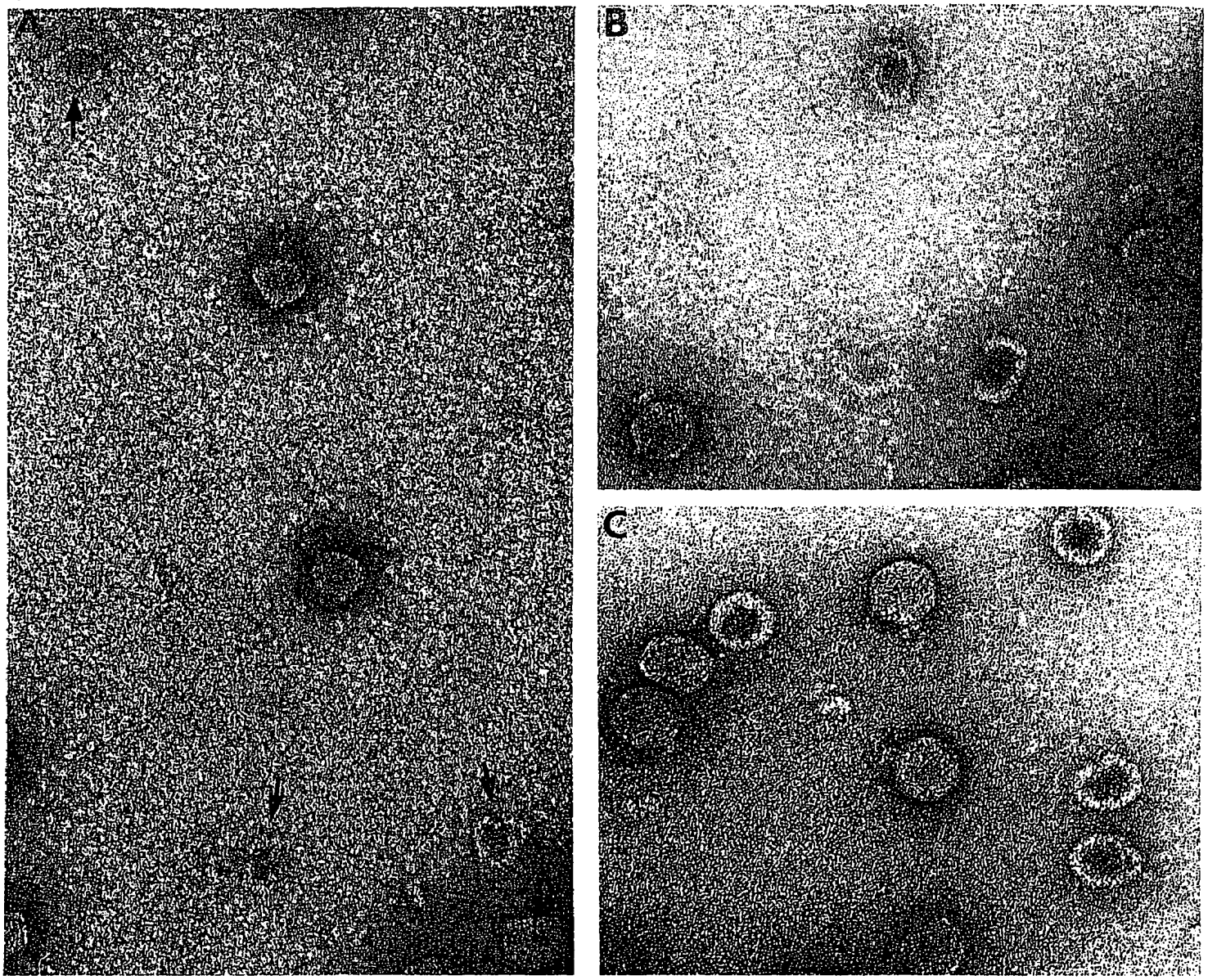

FIGURE 4 Electron microscopy of intermediates. Coat and scaffolding protein were mixed together at a final concentration of 0.77 mg each, and the reaction was allowed to proceed for various times. The reaction mixture was then treated with a final concentration of 0.73% glutaraldehyde for 20 s. The assembly reaction was then stopped by 10-fold dilution with buffer B. 20 μl of reaction mixture was mixed with 10 μl of P22 phage counting standard, and a grid was made for negative stain. Because of the need to get early time points close together, this procedure was followed independently for each time point. *A*, 30 s; *B*, 60 s; *C*, 120 s.

concentration of coat protein remaining in the supernatant appeared to be constant at an average value of ~0.3 mg/ml. This is consistent with an assembly mechanism in which the first step involves the formation of a nucleus of coat protein.

Initiation of procapsid assembly in vitro

To identify the oligomeric species involved in nucleation, we examined the initial rate of assembly at low coat protein concentration where scaffolding protein could be provided in excess. Scaffolding protein concentration was held constant at approximately fourfold excess, and the reaction was monitored at 250 nm to increase the scattering signal. At coat protein concentrations between 0.3 and 0.5 mg/ml, the reactions displayed a lag phase, followed by an increase in the turbidity (Fig. 6, *A* and *B*). At coat protein concentrations >0.5 mg/ml, the lag phase was not apparent (Prevelige et al., 1988; Thomas and Prevelige, 1991). The presence of a lag phase provides additional evidence for a nucleation limited reaction. Since assembly is likely to be a multistep reaction

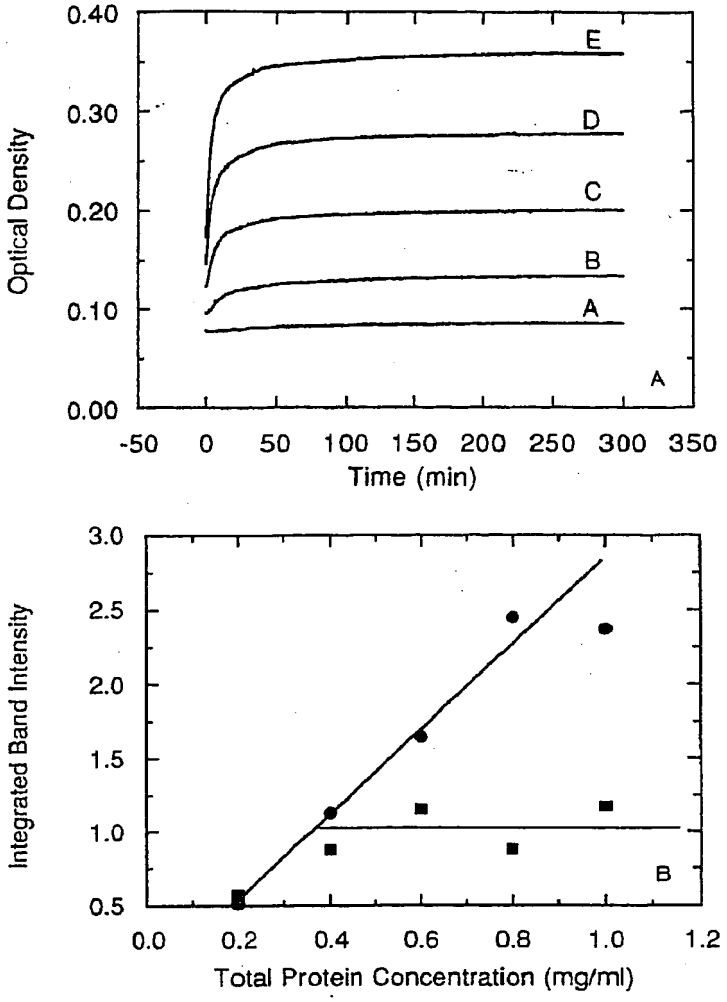

FIGURE 5 Determination of the critical concentration. Assembly reactions were performed in the presence or absence of 1 mg/ml scaffolding reaction at 20°C. (*A*) The turbidity was monitored for 5 h at 350 nm, and coat protein concentration was *A*, 0.2 mg/ml; *B*, 0.4 mg/ml; *C*, 0.6 mg/ml; *D*, 0.8 mg/ml; and *E*, 1.0 mg/ml. (*B*) After 5 h the reactions were centrifuged to pellet procapsids and the coat protein content of the supernatant fraction determined by quantitative gel electrophoresis. Reactions are shown lacking scaffolding protein (●) and containing scaffolding protein (■).

involving two component proteins, it is possible that the rate-determining step could change with subunit concentration. For this reason the kinetic measurements were confined to the concentration regime where a lag phase was apparent.

For a nucleation-limited reaction, the size of the nucleation complex may be determined by examination of the dependence of the reaction on protein concentration. The methodology used for single component systems involves determining the $t_{1/2}$ of the reaction as a function of concentration or alternatively fitting the entire progress curve (Oosawa and Kasai, 1962; Wegner and Engel, 1975; Frieden and Goddette, 1983; Tobacman and Korn, 1983; Voter and Erickson, 1984). For a two component system (i.e., coat and scaffolding protein) in which the concentration dependence of both species is unknown, one component can be held in gross excess throughout the entire reaction, effectively eliminating it from the analysis (Frost and Pearson, 1961). Since we could not generate the quantities of biologically active protein required for this approach, we instead maintained a modest excess of scaffolding protein and utilized data only from very early times during the reaction when the scaffolding protein concentration was relatively unchanged. We have followed a similar approach when examining the concentration dependence of the scaffolding protein described below.

The initial rate of assembly was determined by fitting tangents to the progress curves at early times (Fig. 6, *A* and *B*). The ln of the initial rate of assembly was plotted against the ln of coat protein concentration (Fig. 6 *C*). The data were well fit by a line with a slope of 4.8. Tangents were repeatedly drawn to this data set using slightly different windows. The slope of the ln vs. ln plot varied from ~4.8 to 5.2. A completely independent determination of the concentration dependence yielded a value of 5.4.

Dependence of rate of assembly on scaffolding protein concentration

To investigate the dependence of early steps in the reaction on scaffolding subunits the rate of development of turbidity was monitored at 250 nm as a function of scaffolding protein concentration (Fig. 7 *A*). The coat protein was held constant at 1 mg/ml, the highest concentration at which it could reliably be produced (Prevelige et al., 1988). Assuming a product stoichiometry of 2 coat:1 scaffolding molecule to maintain at least a fivefold excess of coat protein, the scaffolding protein could only be examined at concentrations below 0.1 mg/ml. A practical lower limit was encountered at 0.05 mg/ml, below which the time-dependent increase in signal was too small to measure accurately. The fact that the reaction proceeded even at scaffolding concentrations as low as 0.05 mg/ml demonstrates that if there is a critical concentration for scaffolding protein it must be lower than 0.05 mg/ml.

Tangents were fit to the initial reaction, and a plot of ln rate vs. ln scaffolding concentration was constructed (Fig. 7 *B*). This experiment was performed four times, each time with a fresh preparation of coat protein. Although the data from any given experiment fell on a line, the slopes obtained ranged between 1.7 and 3.5. Although the total coat and scaffolding protein concentration was known accurately, there were likely to be small differences from preparation to preparation in the relative activity of the coat protein toward assembly. The variability in scaffolding protein concentration dependence may reflect changes in the relative contributions

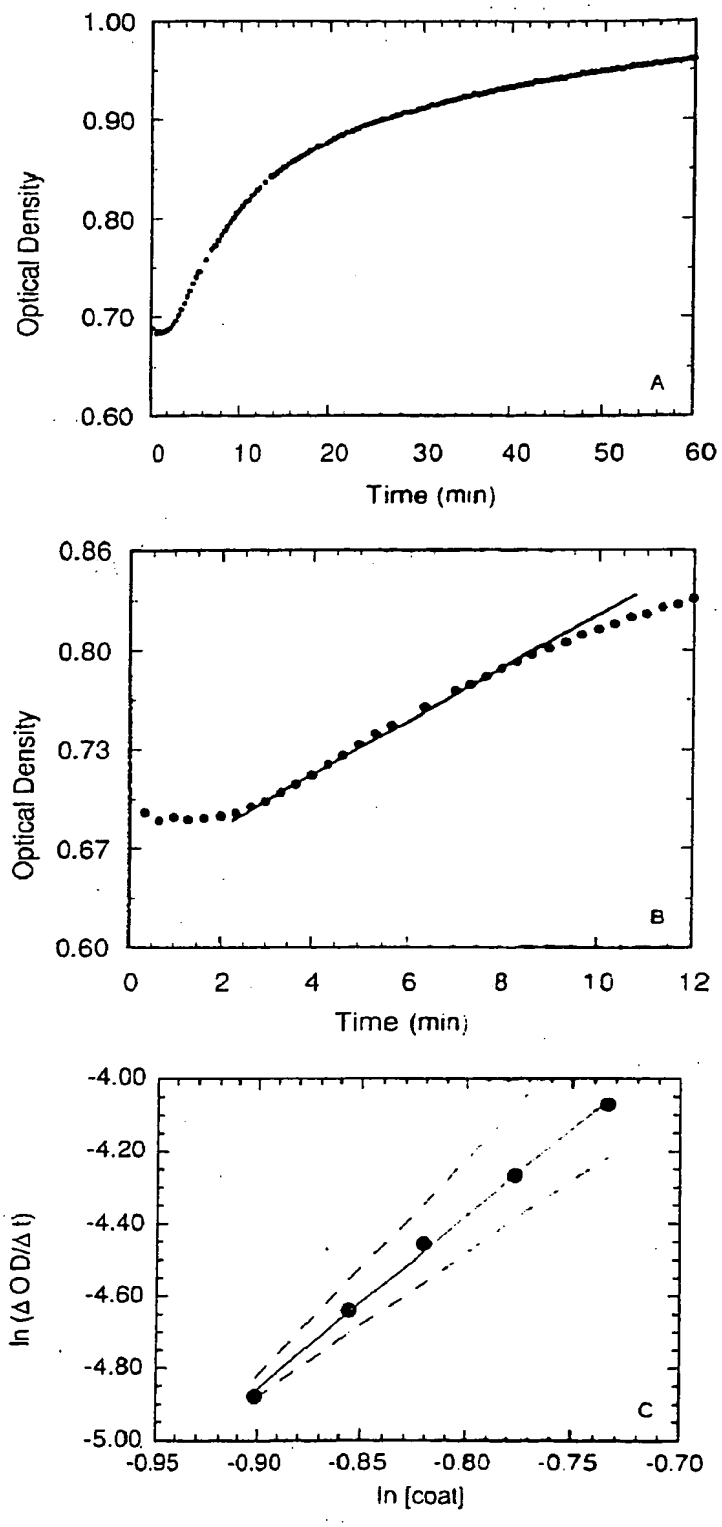

FIGURE 6 Dependence of rate of assembly on coat protein concentration. The overall progress curve (*A*) and initial rate region (*B*) for the assembly reaction with a coat protein concentrations of 0.480 mg/ml. Reactions were also performed with final coat protein concentrations of 0.406, 0.425, 0.440, and 0.460 mg/ml. Tangents were drawn by hand to the initial rise regions for all progress curves (as illustrated in *B*), and the slope was determined. The slopes of a reasonable family of lines through the initial region were determined at each concentration. The particular family of lines used had a minimal effect on the overall order of the reaction determined. (*C*) The ln slope vs. ln [coat protein] is plotted. The points are well fit by the indicated line that has a slope of 4.8. The dotted lines represent slopes of 6 (*upper*) and 4 (*lower*). Scaffolding protein concentration was held constant at 1 mg/m; turbidity was monitored at 250 nm in a 1-cm cell. Data was collected at 20-s intervals for ~1 h.

of the nucleation and growth steps to the rate of the overall process over a very narrow range of effective coat concentration. The results nonetheless suggest that scaffolding protein oligomers are required for the early steps in assembly.

DISCUSSION

There are some important distinctions between the models for helical polymerization and a reasonable model for icosahedral polymerization. Models of helical polymerization are generally based on the repeated addition of a single subunit to a helical structure in a process that is in principle indefinite. It is reasonable to assume that this addition occurs at a single growing point located at the tip of the growing helix and that, apart from the case of breakage of the growing polymer, the number of potential sites for growth is not increased by the growth phase itself. For the polymerization of helical structures, it is generally not necessary to consider the possibility of a change in the rate-determining step in growth as a function of monomer concentration, although evidence for such a change can be seen in the work of Asakura (1968) on the seeded polymerization of flagellin.

In contrast, the assembly of procapsids requires both coat and scaffolding proteins, and the addition of both species must be considered. The procapsid has the coat protein subunits arranged on a T = 7 lattice composed of 60 pentavalent and 360 hexavalent coat protein subunits (12 pentamers, 60 hexamers) (Prasad et al., 1993). The procapsid also contains ~300 scaffolding protein molecules. A simple scheme involving the repeated addition of identical mixed oligomers will not result in the formation of a procapsid. Therefore, it seems reasonable to postulate that the growth phase be composed of nonequivalent steps and require the ordered addition of nonequivalent subunits or oligomers. In this case, a protein concentration–dependent change in rate-determining step is possible. For example, in the case where growth proceeds via the addition of coat protein hexamers and pentamers that are in equilibrium, high protein concentration will favor hexamers, thereby making the addition of pentamers limiting, whereas at low concentration addition of hexamers will be limiting. The addition of subunits to the growing structure can potentially result in an increase in the number of growing sites as the structure approaches a hemisphere with continued addition of subunits, resulting in both a decrease in the number of po-

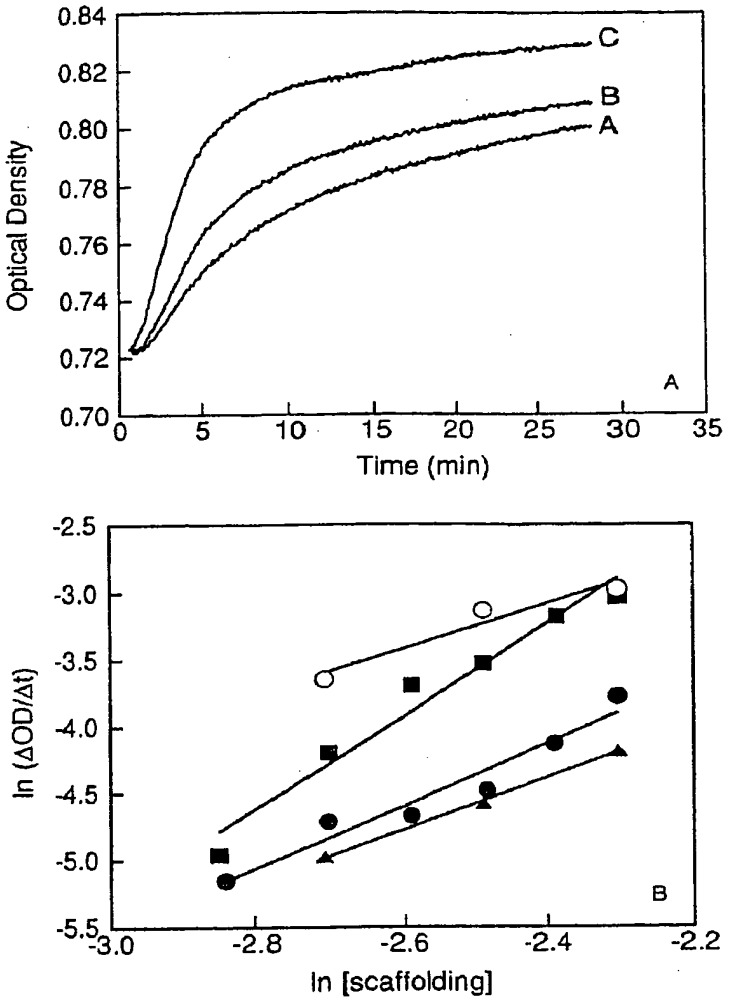

FIGURE 7 Dependence of rate of assembly on scaffolding protein concentration. Assembly was performed as in Fig. 3 except that coat protein concentration was held constant at 1 mg/ml and the scaffolding protein varied over the range 0.03–0.1 mg/ml and data was collected at 3-s intervals. This experiment was performed four times with different solutions of coat protein. (*A*) Progress curves from a typical experiment. Scaffolding protein concentration was *A*, 0.067 mg/ml; *B*, 0.084 mg/ml; and *C*, 0.100 mg/ml. Tangents were drawn to the region of initial rise (the first 5 min), and the slope was determined as in Fig. 3. (*B*) The ln slope vs. ln [scaffolding protein]. The points were fit by linear least-squares procedure that yielded the lines shown. The slopes range between 1.7 and 3.5.

tential growing sites and increased steric constraints for subunit addition as the structure approaches closure. These considerations make the construction of a detailed kinetic model a formidable task, and therefore we interpret our results only qualitatively in terms of a reasonable model of assembly.

The assembly pathway is well directed

The assembly reactions were followed by turbidity and electron microscopy. These experiments demonstrate that the primarily contribution to the turbidity is from the fully assembled shells in solution. The lack of a significant population of partially assembled intermediates suggests that growth is fast relative to initiation and provides evidence for a nucleation limited assembly reaction.

All the polymerized forms observed could be related to presumed morphological intermediates in the assembly reaction. This suggests that there is a well-defined assembly pathway that leads directly to product, rather than polymerization via a random search in which subunits are added and removed in various configurations until the structure forms a closed shell, the presumed minimum energy state.

Assembly follows a nucleated polymerization mechanism

Nucleated polymerization is a consequence of a series of less favorable interactions between subunits, giving rise to a stable seed to which subunits then add. The addition of subunits to the seed is a favorable process. A thermodynamic consequence of the need to populate unstable oligomers is the appearance of a critical concentration, below which no assembly occurs and above which monomer and polymer are in equilibrium (Oosawa and Kasai, 1962; Oosawa and Asakura, 1975; Erickson and Pantaloni, 1981). In the case of procapsid assembly, the critical concentration is ~0.3 mg/ml coat protein. Since procapsids are stable once they are formed, there is not a true equilibrium between monomer and polymer but rather a kinetic trap. This should not affect the interpretation of the results in terms of the critical concentration required for assembly. The methodology used, centrifugation and SDS-gel electrophoresis, does not allow us to demonstrate that the soluble coat protein is strictly monomeric. However, electron microscopy of assembly reactions at late times argues against the presence of populated oligomers. Using the presence of assembly as an indicator, no critical concentration was observed for the scaffolding protein.

In at least some cases, icosahedral capsid assembly proceeds by the association of preformed capsomeric substructures such as hexamers and pentamers. The papilloma viruses SV-40 and polyoma are composed of 72 pentamers arranged in a T = 7 lattice. Of the 72 pentamers of the coat protein, 60 are hexavalent and 12 are pentavalent (Liddington et al., 1991; Rayment et al., 1982). The major coat protein of polyoma virus VP1 has been expressed in *Escherichia coli* and spontaneously forms pentamers, and these pentamers could in turn polymerize into properly dimensioned capsids (Salunke et al., 1986). These results suggest that for polyoma, virus assembly can proceed from preformed pentamers. We believe that this is an unlikely pathway for P22 procapsid assembly. In considering the construction of a T = 7 icosahedron from preformed pentamers and hexamers, it is difficult to imagine a growth pathway where

the addition of either a single pentamer or hexamer would dramatically stabilize the growing structure, and thus a mechanism of this type is unlikely to show strongly nucleated assembly.

We prefer a model in which monomers or small oligomers in solution assemble sequentially to form a nucleation complex, such as the association of five monomers to form a pentamer. This structure would act as the seed, and subunits could then add rapidly to the growing structure. This model would be expected to show strongly nucleated behavior, as observed for procapsid assembly.

The presence of a concentration barrier to nucleation could pose problems in vivo. In vivo, the portal protein, pilot protein, phage messenger RNA (Bazinet et al., 1990), and cellular factors may participate in initiation. Thomas and Prevelige (1991) found that the presence of a small number of subunits of the gene 16 pilot protein in the assembly reaction dramatically increased the rate of assembly in vitro. These subunits interacted directly with coat protein in solution in a manner suggestive of their binding a weakly populated oligomer. Gene product 16 subunits also lowered the critical concentration of coat protein required for assembly (Thomas, D., and P. E. Prevelige, unpublished data). This protein may perform a similar role in vivo.

The nature of the nucleation complex

The turbidity curves at low coat protein concentration appeared sigmoidal. A sigmoidal progress curve means that the rate of the reaction is increasing with time, due to an increase in the concentration of reactive species as a function of time. This could result from either (*a*) an increase in the number of reactive sites for the subunits to bind to, as, for example, by the accumulation of nucleation complex and partially assembled structures, perhaps with a contribution from an increase in the number of binding sites in the growing structures, or (*b*) the accumulation of reactive subunits, capable of being incorporated into the growing polymer, as, for example, by the formation of a coat/scaffolding dimer or a scaffolding protein induced conformational change in the coat protein. The latter mechanism does not account for the critical concentration, and thus we prefer the former.

The dependence of the rate of assembly on coat protein concentration was fifth order over the range of concentrations tested. Before attempting to interpret this result, it is useful to examine the assumptions underlying the methodology. The first assumption is that turbidity is a valid measure of the rate of assembly. Using purified procapsids, we have demonstrated that the turbidity is directly proportional to their number in the concentration range used here. The correlation between the turbidity and the appearance of particles by electron microscopy, as well as the result of the dilution experiment, suggests that there are very few intermediates present during the course of the assembly reaction. Thus, we believe that the approximation that rate of development of turbidity reflects the rate of procapsid formation is valid.

A second assumption is that the reaction is zero order with respect to scaffolding protein. In kinetic analysis, this condition is generally approximated by supplying one component in gross excess. It was not possible to purify enough scaffolding protein to satisfy this requirement. An alternative approach is to utilize data from very early times where the concentration of the components is essentially unchanging. In an attempt to meet these conditions, we provided a fourfold excess of scaffolding and only used data from very early times in the assembly reaction.

For the polymerization of actin, fitting the entire progress curve at a variety of protein concentrations is the preferred way to determine the size of the nucleation complex. Because we cannot make the reaction zero-order in scaffolding protein over the entire time course and because we do not yet know the nature of the growth steps, we cannot produce a sufficiently detailed model. For this reason, we confine our interpretation to a very simple model using only the initial rate data.

The assembly reaction displays a strong dependence on coat protein concentration, which is best fit as a fifth-order dependence. The high concentration dependence provides additional evidence for the nucleation limited character of the reaction. The fifth-order dependence of the assembly reaction derived from the coat protein-concentration dependence is consistent with a model in which the rate-determining nucleation step is the formation of a transient pentameric complex of coat protein that is subsequently stabilized by interaction with scaffolding protein to form the initiation complex. An alternative interpretation analogous to models for single component polymerization (e.g., tubulin, actin) is that nucleation requires formation of a tetramer, followed by growth through the addition of coat protein monomers (Oosawa and Asakura, 1975). This model does not include the known requirement for scaffolding protein in the polymerization pathway nor is there any fourfold symmetry in the final procapsid structure (Earnshaw et al., 1976; Casjens, 1979; Prasad et al., 1993). For these reasons, we prefer the pentamer model. A pentameric initiation complex could form a vertex in the growing procapsid and is appealing because the pentameric vertices of subtriangluated icosahedra are the only structural units in which the conformation of all the constituent subunits are identical. It is not known whether the alternative conformations of the subunits preexist in solution or are generated upon assembly. If they preexist in solution, then productive assembly that initiated at other than a vertex would require not only that the appropriate

number of subunits associate but also the appropriate types.

Rate-limiting nucleation from a pentamer of coat protein subunits requires that the pentamer not be a stable populated species in solution. We have been unable to detect any coat protein oligomers in solution by sucrose gradient centrifugation (Fuller and King, 1982; Prevelige et al., 1988). Preparations of coat subunits similar to those used in these experiments also have been analyzed by analytical ultracentrifugation using both sedimentation velocity and equilibrium techniques. Although the coat protein slowly polymerizes at high protein concentration, no evidence for any populated coat protein oligomers in solution was detected (Laue, T., personal communication).

The role of the scaffolding protein

The formation of procapsids depends on the presence of scaffolding protein. Our results suggest that a trimer or dimer of scaffolding protein is involved in the nucleation or growth phase of the assembly reaction. Experiments in the analytical ultracentrifuge provide evidence for a monomer ↔ trimer equilibrium for scaffolding subunits in solution (Yphantis et al., 1990), suggesting that the trimer may be an active form.

The slopes obtained for the initial rates of the assembly reaction at limiting scaffolding subunit concentrations probably contain contributions from both nucleation and growth phases. If these steps display different dependences on scaffolding protein, then the slopes determined experimentally will reflect contributions from both terms. Since nucleation is fifth order in coat protein concentration, whereas growth is not, slight variations in effective coat protein concentration would alter the relative contribution from these two steps and may explain the variability observed in these determinations.

When does subunit switching occur?

A problem in the construction of icosahedral shells is determining how and where the alternative conformations needed for shell construction are generated (Caspar and Klug, 1962; Caspar, 1980; Rossmann, 1984). Studies of the in vitro assembly of brome mosaic virus in the absence of RNA (Berthet-Colomais et al. 1987) and turnip crinkle virus in the presence of RNA (Sorger et al., 1986) have demonstrated that these T = 3 icosahedral shells grow by the progressive addition of small building blocks, probably coat protein dimers, to the growing shell. For the in vitro assembly of brome mosaic virus, the rate of subunit incorporation depended on the frequency of subunit collisions. Since it is thought that dimer switching occurs on the growing shell (Silva and Rossmann, 1987), this result suggests that the subunit switching is fast if it occurs at a single growing site.

The studies reported here on the assembly of procapsid shells utilize capsid subunits that exhibit the regulated polymerization observed in vivo (Prevelige et al., 1988; Thomas and Prevelige, 1991). Although a coat protein shell lacking scaffolding protein is stable, the coat protein subunits are not capable of self-polymerization, indicating that the dominant solution state is an unassociable form (Prevelige et al., 1988). The conformational switching involved in passing from unassociable to associable forms presumably takes place on interaction with the scaffolding subunits, either in solution or on the edge of the growing shell. If the active species in shell growth is a coat monomer, then the switching that generates the different positional isomers of the coat subunits within this icosahedral lattice may be coupled to the conformational activation process. In this case, both activation and determination of bonding potential would occur on the edge of the growing structure and not in solution.

The pathway for polymerization of P22 subunits differs from the pentamer association model for picornaviruses (Rueckert et al., 1969; Rueckert, 1991) and papovaviruses (Salunke et al., 1986; Liddington et al., 1991) and the dimer association model for the T = 3 RNA containing plant viruses (Rossmann and Johnson, 1989; Silva and Rossmann, 1987). These capsids are apparently assembled by the incorporation of oligomers formed in solution that correspond to defined structural elements of the mature capsid. The relative complexity of the assembly of P22 procapsids may be due to the requirements of building an active structure capable of packaging DNA and subsequently transforming into a stable delivery vehicle.

We thank T. Laue of the University of New Hampshire and D. Yphantis of the University of Connecticut for communication of unpublished work. We thank B-l. Chen, C. Gordon, C. Teschke, B. Greene, and L. F. Kolakowski for helpful discussion and C. Frieden for helpful comments.

This work was supported by National Institutes of Health grant GM-17980 (J. King) and GM-47980 (P. E. Prevelige).

Received for publication 14 September 1992 and in final form 9 November 1992.

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Viral shell game

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The basic structure of the capsid shells of "spherical" viruses was first described in detail by Caspar and Klug (1), who proposed a geometrical model in which the triangular faces of an icosahedron are imagined to be "triangulated" into smaller triangles, each of which is then fitted with three identical protein subunits to form the lattice of the capsid. Only certain integral multiples of 60 subunits (the "triangulation numbers", T) are allowed. In the resulting structures, the subunits are arranged "quasi-equivalently": that is, each subunit makes approximately the same bonding contacts with its neighbors as do all the other subunits, but there are small distortions in the subunit interfaces or the subunits' shapes to allow them to accommodate to the T geometrically slightly different environments that they find themselves in. An alternative way to visualize the predictions of the Caspar-Klug theory is to think of the shell as having a cyclic pentamer of the subunit at each of the 12 vertices of an icosahedron, with the space between the pentamers filled in by cyclic hexamers, the number and positioning of which is characteristic of the triangulation number.

Over the three decades since this theory was proposed, most of the variations on its basic themes that might have been imagined (as well as one that was probably never imagined) have been documented: some viruses use a separately encoded protein to occupy the fivefold corners; in some viruses a single polypeptide occupies the position expected to be occupied by two quasi-equivalent subunits; and in the beautifully grotesque structure of the papova viruses all the capsomeres (including the 6-coordinated ones) are pentamers equipped with flexible bonding arms to compensate for the broken symmetry (2). Moreover, in most of the available atomic resolution structures of mature viruses, it would be a stretch to call the inter-subunit bonding quasi-equivalent (though since the capsid subunits of many viruses undergo substantial conformational changes following shell assembly, it is not clear that nonequivalences in the mature virion reflect nonequivalences in the initially assembled shell). Despite its weathering over the years, the Caspar-Klug theory remains a useful way to think about virus structure, and there are many examples, including the P22 prohead shells discussed below, which follow the simple Caspar-Klug model in an apparently uncomplicated way.

Inherent in the idea that identical subunits can occupy similar but non-identical positions in the lattice is that those same subunits, with their exact geometries differently assorted, should be able to form shells of a wide variety of sizes and shapes. That protein subunits of viral capsids do indeed have such potential is known from numerous examples. Thus, the capsid subunit of bacteriophage P2, which normally makes $T = 7$ shells (12 pentamers and 60 hexamers), can be efficiently subverted into making $T = 4$ shells (12 pentamers and 30 hexamers) under the genetic direction of a co-infecting bacteriophage P4. Bacteriophage T4, which normally has a prolate capsid based on an elongated $T = 13$ icosahedral structure, can find itself with an isometric "petite" capsid or a super-elongated "giant" in the presence of any of a variety of mutations affecting either the subunit itself or one of several assembly accessory proteins. Despite the potential for incorrect assembly, the frequency of error in assembly during the normal course of an infection by a wild-type virus is remarkably low; evidently the protein subunits are either directed toward the correct structure or away from the many possible incorrect ones.

None of the elegant structural information available for mature virions gives more than an occasional hint about the mechanisms by which protein subunits are assembled into these shells inside the cell. What are the intermediates in assembly: do subunits first assemble into oligomeric units or do they add individually to the growing shell? How does an individual subunit "know" whether it should assemble as part of a five- or sixfold unit, or stated differently, how is it determined where the pentamers are inserted into the lattice of hexamers to determine the size and shape of the shell? Is all the information to specify accurate assembly contained in the subunits themselves, or are other factors (e.g., the viral nucleic acid or other proteins) needed?

The paper by Prevelige, Thomas, and King in this issue takes a small but nicely shaped bite out of this daunting set of questions, using bacteriophage P22 of *Salmonella typhimurium* as the experimental model. P22, as a typical example of the dsDNA phages, has the interesting property that assembly of the shell requires an abundant second protein, the "scaffolding" protein, in addition to the "coat" subunit that will make up the mature shell. Coat and scaffolding subunits co-assemble into a structure called a prohead with a $T = 7$ icosahedral shell of 420 coat subunits surrounding an internal core (the scaffold) of ~300 scaffolding proteins. (The scaffolding subunits subsequently leave the structure as DNA is pumped in, probably by slithering out through holes in the prohead shell.) These authors have shown that P22

0006-3495/93/03/836/02 $2.00

proheads can be assembled in vitro using only purified coat and scaffolding protein (3, 4). Either protein alone is stable in solution as a soluble monomer, but when the two are mixed, they assemble rather efficiently into normal-appearing scaffold-containing proheads. The system is now well enough under control that it is possible to make quantitative kinetic measurements of the assembly process and to search for assembly intermediates, and these studies are described in the current paper.

Measuring shell assembly by turbidity and by electron microscopy, the authors show that the reaction appears to be nucleation limited, with fast completion of assembly following successful nucleation. This means that assembly intermediates are scarce, but it is still possible to identify some probable intermediates. These all fit a picture in which assembly initiates at one point in the shell and the shell grows progressively from that point by addition of subunits to the edge. Significantly, there is no indication of any protein subassemblies that form before adding to the growing shell; most likely the coat subunits add individually to the edge of the shell. The variation of initial rate of assembly with protein concentration shows that nucleation is fifth order in coat subunit and second or third order in scaffolding protein. The simplest interpretation of these numbers is that the nucleation complex is a pentamer of the coat protein (presumably one that will form one of the corners of the assembled shell) stabilized by a few copies of the scaffolding protein.

The fascinating question of how the scaffolding protein helps the coat protein to assemble correctly is still largely unanswered, though the clearer view of the overall process afforded by these studies makes it easier to formulate sensible hypotheses. The coat protein needs to undergo two sorts of conformational adjustments or switches as it goes from the soluble monomeric state to being assembled in a shell: it must change from the unassociable form of the solution to the associable form of the shell, and each subunit must adjust to one of the seven quasi-equivalent positions available in the shell lattice. Previlege et al. suggest that both these changes in the protein may take place at once, at the edge of the shell, under the influence of the scaffolding protein. This view appears to make the best use of the currently available data, though exactly what the scaffolding protein might be doing in this process is still unclear, particularly since the non-integral ratio of coat to scaffolding proteins in the assembled prohead argues against a simple one-to-one interaction between the two proteins. More experiments remain to be done.

Is the P22 assembly mechanism that is gradually emerging a universal mechanism of capsid assembly? Evidently not, since work on various other viruses argue for mechanisms that are clearly incompatible with the P22 data, such as assembly from pentamers or nucleation on the viral nucleic acid (5–7). Certainly the P22 mechanism will be invaluable for understanding how other viruses that use scaffolding proteins achieve accurate assembly. Somewhat surprisingly from the point of view of the P22 work, another lambdoid phage, HK97, appears to assemble its prohead without the assistance of a separate scaffolding protein. Instead, the HK97 subunit has a stretch of 100 amino acids at its amino terminus that is needed for correct assembly and then is cleaved off, and it may provide an analogous assembly-aiding function to that provided by the scaffolding protein in P22. HK97 also differs from P22 in that pentamers and hexamers of the subunit are intermediates in assembly (Xie, Duda, and Hendrix, unpublished results). Apparently these two phages, which are very closely related by other criteria, use quite different mechanisms to assemble very similar structures. Each of the different assembly mechanisms used by different viruses will inform our understanding of the sorts of dances proteins are capable of in achieving these complex and highly ordered structures.

Received for publication and in final form 17 December 1992.

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